Correlation of apoptosis with change in intracellular labile Zn(II) using Zinquin [(2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II)

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Zinquin [(2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)-acetic acid], a membrane-permeant fluorophore specific for Zn(II), was used with spectrofluorimetry and video image analysis to reveal and quantify labile intracellular Zn. Zinquin labelled human chronic-lymphocytic-leukaemia lymphocytes, rat splenocytes and thymocytes with a weak diffuse fluorescence that was quenched when intracellular Zn was chelated with *NNN'N'*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) and was greatly intensified by pretreatment of cells with the Zn ionophore pyrithione and exogenous Zn. There was substantial heterogeneity of labile Zn among ionophore-treated cells, and fluorescence was largely extranuclear. The average contents of labile Zn in human leukaemic lymphocytes, rat splenocytes and rat thymocytes were approx. 20, 31 and 14 pmol/10^6 cells respectively. Morphological changes and internucleosomal DNA fragmentation indicated substantial apoptosis in these cells when the level of intracellular labile Zn was decreased by treatment with TPEN. Conversely, increasing labile Zn by pretreatment with Zn plus pyrithione suppressed both spontaneous DNA fragmentation and that induced by the potent apoptosis-induced agents colchicine and dexamethasone. These results suggest that prevention of apoptosis is a function of labile Zn, and that a reduction below a threshold concentration in this Zn pool induces apoptosis.

INTRODUCTION

The essential role of Zn(II) in the survival, growth and metabolism of unicellular and multicellular organisms is partly explained by the requirement of metalloenzymes, Zn-finger transcription factors and hormones for this element (Vallee and Galdes, 1984; Williams, 1984; Coleman, 1992). Zn may be involved in regulation of cell numbers by its roles in both proliferation and death by apoptosis. Zn is essential at several points of the mitotic cycle, where it may interact reversibly to induce synthesis of thymidine kinase, activate DNA polymerase and take part in spindle formation (Chesters, 1989). High concentrations of extracellular Zn suppress apoptosis in many *in vitro* models, but complete suppression is also obtained with a physiological extracellular concentration (25 μM) provided a Zn ionophore, such as sodium pyrithione, is present to facilitate the cellular uptake of Zn (Zalewski et al., 1991). In addition, deprivation of cellular Zn induces apoptosis, both *in vitro* and *in vivo* (Elmes, 1977; Martin et al., 1991; Zalewski et al., 1991].

To investigate these functions of Zn it is essential to reveal and quantify the metabolically important pool intracellular Zn. As total cellular Zn is decreased only slightly in cases of severe Zn deficiency, while cell function is profoundly disturbed, the important fraction of Zn probably resides in a relatively small pool that exchanges its content of Zn with both intracellular and extracellular pools (Bettger and O'Dell, 1981; Chesters, 1989). Most of the cellular Zn detected by atomic-absorption spectroscopy is very tightly bound to cellular proteins and essentially non-exchangeable. Studies of intracellular free Ca^{2+} and some other biologically important ions have been greatly facilitated by the use of fluorophores which are loaded into cells and fluoresce specifically in contact with the ion; for example, Ca^{2+} has been detected and measured using Quin-2, 1,2-bis-(1-aminophenoxy)-ethane-*NNN'N'*-tetra-acetic acid (BAPTA) analogues, Fura-2, Indo-1, Fluo-3 and other compounds, each with their own particular advantages and disadvantages (reviewed by Tsien, 1989). These compounds have been loaded into cells as esters which are cleaved by intracellular esterases to yield negatively charged fluorophores that are effectively trapped within the cells. Analogous compounds for the detection of Zn have not been available. Previously, toluenesulphonamidoquinoline has been used as a specific fluorescent histochemical stain for Zn in tissue sections of brain, heart and some other tissues (Frederickson et al., 1987; Savage et al., 1987; Frederickson, 1989; Fliss, et al., 1990). However, this compound has not been applied successfully to measure Zn in living cells. We have synthesized various compounds containing sulphonamidoquinoline and one or more esters. One of these, Zinquin [(2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)acetic acid], contains an ethyl ester in place of the 6-methoxy group of toluenesulphonamidoquinoline, facilitating its retention in living cells. Zinquin reacts with Zn to give a strong fluorescent signal. Here we have used Zinquin to correlate changes in content of labile Zn, induced by either a Zn chelator or a Zn ionophore, with susceptibility of chronic-lymphocytic-leukaemia (CLL) cells and other lymphoid cells to undergo apoptosis.

MATERIALS AND METHODS

Materials

Zinquin and its ethyl ester (AMRAD Corporation, Kew, Victoria 3101, Australia) were stored at 5 mM in dimethyl sulphoxide at

Abbreviations used: CLL, chronic lymphocytic leukaemia; *F*, fluorescence by spectrofluorimetry; $F_{max.}$, maximal fluorescence; $F_{min.}$, minimal fluorescence; FBS, fetal bovine serum; HBSS, Hanks balanced salt solution; *I*, fluorescence by image analysis; TPEN, *NNN'N'*-tetrakis-(2-pyridylmethyl)ethylenediamine; Zinquin, (2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)acetic acid; BAPTA, 1,2-bis-(1-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid.

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−20 °C. Other reagents and their suppliers were $ZnSO_4,7H_2O$ (BDH), colchicine, sodium pyrithione, digitonin, TPEN [*NNN'N'*-tetrakis-(2-pyridylmethyl)ethylenediamine] (Sigma) and dexamethasone (David Bull Laboratories, Vic., Australia). Ultra-pure water (Permutit) was used for making solutions.

Preparation of cells

Thymocytes and splenocytes were prepared from Sprague–Dawley rats maintained on normal Zn-sufficient diets (7.6 mg/100 g). All animal procedures were approved by The University of Adelaide Animal Ethics Committee. Immediately after the rats had been killed, spleen or thymus was removed and cell suspensions made by teasing tissue apart in Hanks balanced salt solution, pH 7.4, containing 1.3 mM Ca^{2+}, 0.9 mM Mg^{2+} and 4.2 mM $NaHCO_3$ (HBSS, Gibco). Contaminating red cells were lysed with Gey's solution. Human CLL cells were obtained from patients undergoing leukapheresis, frozen at a rate of minus 1 °C/min in the presence of 10% dimethyl sulphoxide, and stored in liquid N_2. Before use, cells were thawed, washed and dead cells removed by Ficoll–Hypaque density centrifugation. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, ICN Biologicals), 2 mM L-glutamine, 24 mM $NaHCO_3$ (BDH), 25 mM Hepes (BDH) and 160 μg/ml gentamycin sulphate (Delta West). Cells were washed and suspended in HBSS. Cell viability was monitored by Trypan Blue dye exclusion and phase-contrast microscopy.

Treatment of cells with Zn ionophore

Cells (5×10^6/ml) were treated for indicated periods of time at 37 °C with 25 μM $ZnSO_4$ (approximate concentration of Zn in human plasma is 15 μM) and the Zn ionophore sodium pyrithione (Forbes et al., 1989). Cells were then washed three times with HBSS to remove extracellular Zn prior to labelling with Zinquin.

Fluorescence measurements with pure Zinquin

Zinquin was added to cuvettes containing HBSS and known amounts of Zn^{2+} or other metal ions. Fluorescence was measured at room temperature in a Perkin–Elmer LS 50 luminescence spectrophotometer. Single excitation and emission spectral peaks were observed at wavelengths of 370 nm and 490 nm respectively. The weak fluorescence of Zinquin alone was greatly increased in the presence of added Zn^{2+}, without change in the optimal excitation or emission wavelengths. Binding constants were determined from titration curves of $ZnSO_4$ with 3 μM Zinquin. Zinquin forms both 1:1 and 1:2 complexes with Zn.

Fluorescence labelling of cells with Zinquin

Cells [$(5–10) \times 10^6$ cells/ml] were incubated with Zinquin (final concn. 25 μM) in HBSS for 30 min at 37 °C. Fluorescence of individual cells was observed by using an Olympus microscope with a u.v. B dichroic mirror for low-wavelength excitation. Fluorescence was quantified using the Video Pro Image Analysis System (Leading Edge Pty Ltd., Adelaide, Australia). Photomicrographs were taken directly or from computer images. For analysis of images, background illumination was subtracted from the readings. Spectrofluorimetric measurements were made with cells suspended at $(2–5) \times 10^6$/ml in HBSS. Fluorescence of unloaded cells (due to autofluorescence and light scattering) was subtracted from readings to derive Zinquin-dependent fluorescence. In some experiments washed cells were lysed in the cuvettes by addition of digitonin to 50 μM, and the released Zinquin was saturated with 25 μM $ZnSO_4$. Fluorescence of unloaded lysed cells was subtracted. The subsequent fluorescence ($F_{max.}$) was used to derive the amount of Zinquin in the lysate using a standard fluorescence curve derived by titration of pure Zinquin in HBSS containing 50 μM digitonin and 25 μM $ZnSO_4$. $F_{min.}$ was derived by further addition of 1M HCl to these cell lysates to quench Zn-dependent fluorescence of Zinquin.

Determination of the labile Zn content of cells

Fluorescence readings by spectrofluorimetry were converted into pmol of Zn/10^6 cells using a standard curve derived by titration of increasing amounts of $ZnSO_4$ into a solution of 3 μM Zinquin until the fluorescence was equivalent to that obtained with Zinquin-labelled cells. The medium for this titration was a solution with an ionic constitution resembling that of lymphocyte cytosol [125 mM K^+/20 mM N^+/1 mM Mg^{2+}, Hepes-buffered to pH 7.05 (Tsien et al., 1982)] and supplemented with 0.1 mg/ml BSA. The concentration (3 μM) of Zinquin was equivalent to that loaded into cells (see the Results section).

DNA fragmentation

DNA fragmentation was assayed by detection of DNA fragments in 15000 *g* supernatants of cell lysates using diphenylamine as described by Zalewski et al. (1991). Ladder patterns of DNA fragmentation were examined by ethidium bromide after extraction of DNA and electrophoresis on 1% agarose as described by Forbes et al. (1992).

Expression of results

Data are expressed as means ± S.D. for replicate samples. Statistical significance was determined by Student's *t* test.

RESULTS

Specificity of Zinquin

Fluorescence of a 2 μM solution of Zinquin in HBSS was increased at nanomolar free Zn^{2+} (added in the form of $ZnSO_4$) and was saturated at 1 μM Zn^{2+}. Fluorescence was enhanced 20-fold by 1 μM Zn^{2+}. Cd^{2+} enhanced fluorescence 5-fold, but this metal is not present to a significant extent in normal biological tissues. None of the other metal ions tested (Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Hg^{2+}, Ag^{2+}, Li^+, Pb^{2+}, Mn^{2+}, Ba^{2+} or Al^{3+}) enhanced fluorescence. Zn-dependent fluorescence of Zinquin was unaffected by a 3000-fold molar excess of other biologically relevant bivalent cations, Mg^{2+} and Ca^{2+}. Cu^{2+} forms a non-fluorescent complex and may quench intracellular Zn-dependent fluorescence, although the cytoplasmic concentration of this metal is thought to be very much lower than that of Zn (Williams, 1984).

Zinquin forms both 1:1 and 2:1 Zinquin–Zn complexes with binding constants of $2.7 \pm 0.4 \times 10^6$ and $11.7 \pm 1.9 \times 10^6$ litre·mol^{-1}. These are sufficiently low to preclude Zinquin from interacting with the tightly bound (binding constants of 10^{12}–10^{13} litre·mol^{-1}) structural Zn in metalloenzymes. However, it is not known whether Zinquin can react with incompletely-ligated Zn such as catalytic Zn in metalloenzymes where only three of the ligand-binding sites of Zn are bound to cysteine or histidine and the fourth is occupied by water (Vallee and Auld, 1990).

Quantification of labile cellular Zn by Zinquin fluorescence

While microscopy showed only weak fluorescence in CLL cells labelled with 25 μM Zinquin (the predetermined optimal concentration) in HBSS for 30 min at 37 °C (Figure 1), the fluorescence could be quantified by digital image analysis (Figure 2a) or by spectrofluorimetry of cell suspensions in cuvettes. Fluorescence of unloaded cells, due to autofluorescence and light scattering, was subtracted from all spectrofluorimetric readings. Fluorescence was unaffected by washing the cells after labelling, indicating that Zinquin was effectively trapped within the cells. Fluorescence measurements obtained with Zinquin were stable over 1–2 h. For quantification of labile cellular Zn, it is important that detection of Zn is not limited by cellular uptake of Zinquin. In some experiments, therefore, the amount of Zinquin loaded into cells was derived from the maximal fluorescence ($F_{max.}$) which was determined after releasing cellular Zinquin into a medium containing excess $ZnSO_4$ by lysing the cells with digitonin (see the Methods section), a detergent which does not interfere with the fluorescence of the Zn–Zinquin complex in aqueous solution. It is unlikely that some Zinquin was in cellular compartments not permeabilized by digitonin, since the addition of 0.1 % SDS did not further increase $F_{max.}$. When lymphocytes were loaded with 25 μM extracellular Zinquin, the ratio of F to $F_{max.}$ was usually between 0.1 and 0.3, depending upon the type of cells and other loading conditions, indicating that the amount of free intracellular Zinquin was in large excess over the amount of Zn-bound Zinquin. Since increasing the loading concentration of Zinquin increased $F_{max.}$ without affecting F, the additional Zinquin taken up by the cells does not interact with Zn. Therefore it is unlikely that the uptake of Zinquin by cells will be a limiting factor in the detection of Zn. The amount of Zinquin in cells loaded with 25 μM extracellular Zinquin was determined from the $F_{max.}$ to be about 0.75 nmol/10^6 cells, corresponding to about 3 μM Zinquin in the cuvette volume. When cells were preloaded with Zn, by treatment with $ZnSO_4$ plus Zn ionophore pyrithione, the $F_{max.}$ obtained with subsequent addition of Zinquin was significantly increased. This suggests that Zinquin equilibrates across the plasma membrane with intracellular Zn; some Zinquin will become trapped by formation of a complex with cellular Zn, while other Zinquin molecules will be trapped following cleavage of the ester group by cellular esterases. However, the mechanism by which Zinquin becomes trapped in cells should not affect the final measurement of Zn. Subsequent addition of HCl to quench the Zn-dependent fluorescence of detergent-permeabilized loaded cells resulted in a fluorescence $F_{min.}$ not significantly different from that of unloaded cells. Although lowering of pH will displace most metals from chelators, the specificity of Zinquin for Zn (see 'Specificity of Zinquin' above) suggests that most, if not all, of the specific fluorescence in cells can be attributed to interaction of Zinquin with Zn.

The amount of cellular Zn detected by Zinquin in freshly obtained cells suspended in Zn-free medium was determined from a standard curve of fluorescence of increasing amounts of $ZnSO_4$ in a solution of 3 μM Zinquin in HBSS. Average contents of labile Zn in CLL cells, rat splenocytes and rat thymocytes were 20.4 ± 8.1 pmol/10^6 cells, 31.2 ± 14.6 pmol/10^6 cells ($n = 76$, 24 rats) and 14.4 ± 7.8 pmol/10^6 cells ($n = 40$, 12 rats) respectively.

Effect of Zn ionophore pyrithione on Zinquin fluorescence and apoptotic DNA fragmentation

Effect on Zinquin fluorescence

When lymphocytes were loaded with Zn by treatment with 25 μM $ZnSO_4$ plus sodium pyrithione for 40 min, their subsequent fluorescence with Zinquin was greatly increased, as detected by fluorescence microscopy (Figure 1). The fluorescence was both diffuse and punctate ('starry-sky') and largely restricted to extranuclear regions. Increase in fluorescence with pyrithione treatment was confirmed by measurement of fluorescence intensities by image analysis (Figure 2). Pretreatment of cells with 25 μM $ZnSO_4$ and 1 μM pyrithione increased the mean fluorescence intensity from 8.6 ± 2.4 to 24.6 ± 7.8 (Figure 2b). A further increase in fluorescence to 31.1 ± 12.5 was seen with 4 μM

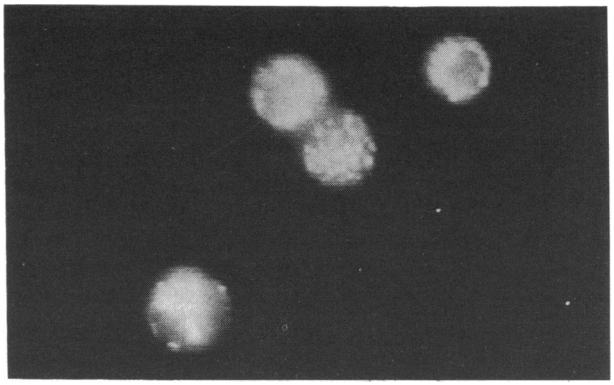

Figure 1 Fluorescence micrographs of pyrithione-treated CLL cells

CLL cells (5×10^6 cells/ml) were incubated in HBSS with 1 μM pyrithione and 25 μM $ZnSO_4$. After 15 min, cells were washed three times with HBSS and Zinquin added to 25 μM. After a further 40 min at 37 °C, cells were centrifuged and resuspended in 100 μl of HBSS and fluorescence was examined by digital image analysis (see the Methods section). Fluorescence micrographs were prepared by directly photographing images from the screen.

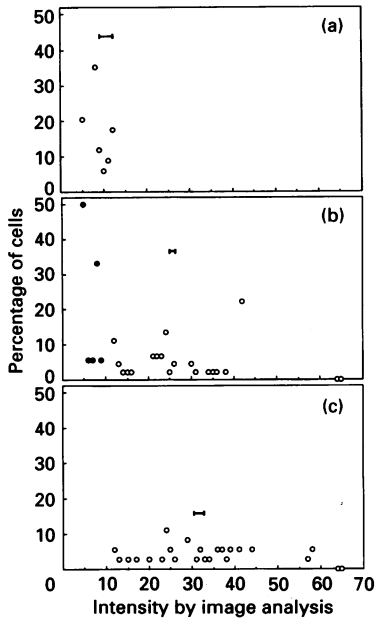

Figure 2 Single-cell fluorescence intensity by image analysis

CLL cells were treated with (**a**) 0, (**b**) 1 or (**c**) 4 μM pyrithione and either 25 μM $ZnSO_4$ (○) or no Zn (●) as in the legend to Figure 1. Fluorescence of individual cells was quantified for approx. 30 cells from duplicate tubes for each treatment. The Figure shows the percentage of positive cells with a given fluorescence intensity. Bars indicate mean intensity (±S.D. for open circles only).

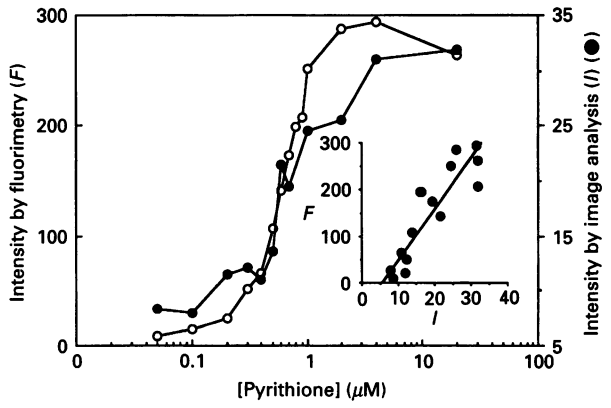

Figure 3 Correlation between fluorescence intensity by image analysis (*I*) and by spectrofluorimetry (*F*)

CLL cells were treated with different concentrations of pyrithione (0–20 μM), in the presence of 25 μM $ZnSO_4$; other conditions were as in the legend to Figure 1. Fluorescence of cells was determined either by spectrofluorimetry (*F*, ○) or by image analysis (*I*, ●). The inset shows correlation between *F* and *I*.

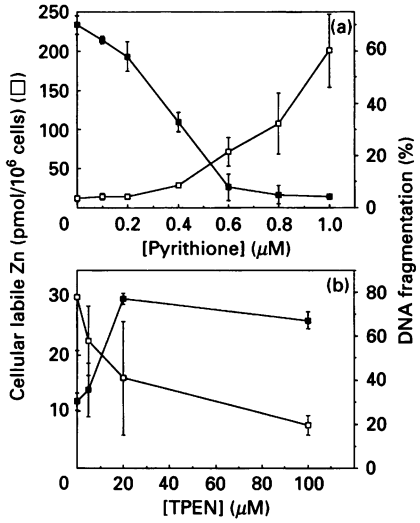

Figure 4 Effect of ionophore and chelator on content of labile Zn and colchicine-induced apoptotic DNA fragmentation

(**a**) Effect of ionophore. CLL cells (10^7/ml) were cultured at 37 °C in RPMI medium containing 10% FBS in the presence of 100 μM colchicine to induce DNA fragmentation and different concentrations of pyrithione (0–1 μM) and 25 μM $ZnSO_4$ to vary the content of labile Zn in the cells. Labile Zn (□) was determined after 40 min of culture by Zinquin-dependent fluorescence of aliquots of cells, while percentage DNA fragmentation (■) was determined in remaining cells after culture overnight. Bars indicate means (±S.D.) of replicate measurements. Results of a typical experiment are shown. (**b**) Effect of chelator. Spleen cells (10^7/ml) were treated with different concentrations of TPEN in RPMI medium containing 10% FBS. Labile Zn (□) was determined after 90 min of culture, while percentage DNA fragmentation (■) was determined in remaining cells after culture overnight. Bars indicate means (±S.D.) of replicate measurements. Results of a typical experiment are shown.

pyrithione (Figure 2c). Higher concentrations of pyrithione had no additional effect. Heterogeneity in Zn labelling was evident both from fluorescence micrographs and digital image plots, even at a saturating concentration (4 μM) of pyrithione (Figure 2c). Figure 3 shows that spectrofluorimetry and image analysis measure similar relative changes in cellular Zn content and that there is a good correlation between the fluorescence values from the two methods (see the inset to Figure 3). The labile Zn content of CLL cells was increased about 15-fold by treatment of the cells with 1 μM pyrithione plus 25 μM $ZnSO_4$. The increase in fluorescence due to treatment with pyrithione was progressively decreased as the Zn content of the medium was decreased, and it was completely prevented by omission of $ZnSO_4$ from the medium (Figure 2b, ●). When experiments were performed in the presence of 10% FBS, there was a small increase in fluorescence in cells treated with pyrithione in the absence of added Zn, owing to the small concentration of endogenous Zn (about 2 μM) already present in the unsupplemented culture medium.

Effect on apoptotic DNA fragmentation

To test effects of preloading cells with Zn on apoptosis, DNA fragmentation was measured in the cells after culture overnight with colchicine, a potent inducer of apoptosis. Colchicine at 100 μM induced morphological signs of apoptosis (decrease in cell volume and pyknotic fragmented nuclei) in most of the cells, and about 60% of the total DNA was fragmented (not sedimenting at 15000 *g* in cell lysates). Electrophoresis showed the DNA ladder pattern typical of internucleosomal fragmentation. At concentrations of pyrithione which increased the labile Zn content of CLL cells, there was a corresponding suppression of DNA fragmentation (Figure 4a). Inhibition of apoptosis was also observed at concentrations of Zn in the medium as low as 5 μM, but progressively decreased at lower concentrations. Only slight inhibition occurred in the absence of $ZnSO_4$, paralleling the slight increase in Zn content. Colchicine-induced DNA fragmentation was almost completely suppressed at a concentration of pyrithione of 0.6 μM. Pyrithione also suppressed spontaneous and dexamethasone (3 μM)-induced DNA fragmentation in rat thymocytes.

Effect of membrane-permeant Zn chelator TPEN on Zinquin fluorescence and apoptotic DNA fragmentation

Effect on Zinquin fluorescence

Pretreatment of lymphocytes (CLL cells, rat splenocytes and rat thymocytes) with the intracellular Zn chelator TPEN quenched subsequent fluorescence with Zinquin. The quenching was dependent on the concentration of TPEN (Figure 4b) and increased with duration of treatment. After several hours of treatment, the fluorescence was not significantly different from that of unloaded cells (results not shown).

Effect on apoptotic DNA fragmentation

Treatment of the lymphocytes overnight with TPEN induced DNA fragmentation and morphological changes of apoptosis in most of the cells. Up to 77% of the total DNA was fragmented (Figure 4b). Although TPEN has a very high affinity for Zn^{2+} and only weakly interacts with Ca^{2+} and Mg^{2+}, it does chelate some other metals such as Cu^{2+} and Co^{2+} (Arslan et al., 1985). Therefore other evidence is necessary to confirm that TPEN causes apoptosis by lowering intracellular labile Zn^{2+}.

Correlation between content of labile Zn and susceptibility of cells to apoptotic DNA fragmentation

There was good correlation between the average content of labile Zn in the cells, as determined by spectrofluorimetry, and the inhibition of DNA fragmentation (Figure 5). Similar results were obtained in CLL cells induced to apoptose by colchicine (Figure 5a) and thymocytes from aged rats which undergo spontaneous

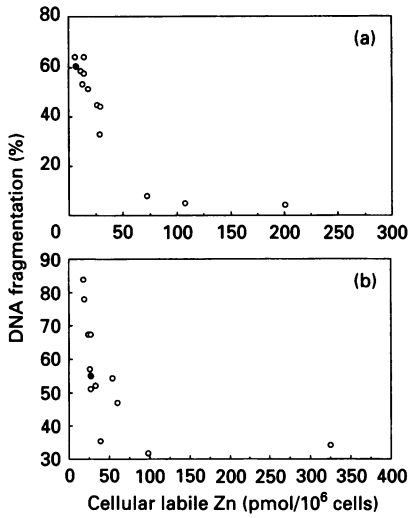

Figure 5 Correlation between labile Zn content and susceptibility or resistance to induction of apoptotic DNA fragmentation

(**a**) CLL cells were cultured in the presence of colchicine, $ZnSO_4$ and various concentrations of pyrithione as described in the legend to Figure 4(a). Percentage DNA fragmentation, after 22 h of culture, is expressed as a function of labile Zn content of cells. ●, control cells treated with colchicine and Zn sulphate, but no pyrithione. (**b**) Thymocytes from 6–9-month-old rats were cultured in the presence of various concentrations of TPEN (0–100 μM) or pyrithione (0–1 μM) plus 25 μM $ZnSO_4$. Percentage DNA fragmentation after 30 h culture is expressed as a function of labile Zn content of cells. ●, Control cells treated without TPEN or pyrithione.

apoptotic DNA fragmentation in extended culture (Figure 5b). In this Figure ● represents cells with normal Zn content. In both cases, there was a steep threshold curve, suggesting that relatively small changes in labile Zn were responsible for large changes in susceptibility of cells to DNA fragmentation. Substantial suppression of DNA fragmentation in both types of cells was evident at labile Zn contents between 50 and 100 pmol/10^6 cells. On the other hand, decreases in labile Zn of only a few pmol/10^6 cells, induced by short-term treatment with TPEN, resulted in large increases in DNA fragmentation (Figure 5b). In these experiments, Zn content was assayed at an early stage of culture, whereas DNA fragmentation was assayed after overnight incubation. Since the Zn content decreases with increasing time of treatment with TPEN, the size of the decrease in Zn required to induce a given amount of apoptosis may be greater than is evident from Figure 5(b).

The correlation between the extent of apoptosis and the level of intracellular labile Zn is consistent with other data implicating Zn in the regulation of apoptosis (Figures 3a and 3b), with the induction of apoptosis *in vivo* in Zn deficiency (Elmes, 1977) and the metal specificity for inhibition of the endonuclease that mediates internucleosomal DNA cleavage (Giannakis et al., 1991).

DISCUSSION

Our studies imply that there is a labile or readily exchangeable pool of intracellular Zn that is important in the regulation of apoptosis. Greatly increased apoptotic cell death in tissues of Zn-deficient animals and in cells deprived of Zn *in vitro*, as well as inhibition by supplemental Zn of cell death induced by apoptotic agents, *in vivo* or *in vitro*, suggest that suppression of apoptosis is a physiological function of Zn (Dinsdale and Williams, 1977; Elmes, 1977; Martin et al., 1991; Zalewski et al., 1991). This labile pool of Zn constitutes only a small part of the total cellular Zn, since most of the Zn in cells is tightly complexed to metalloenzymes and not readily exchangeable (Bettger and O'Dell, 1981). In fact, it has often been difficult to detect a decrease in total cellular Zn in tissues of several Zn-deficient animals by the usual method of atomic-absorption spectroscopy. Where decreases have been detected, they have been small. It has been estimated from ^{65}Zn-radiolabelling studies that the exchangeable compartment of Zn in erythrocytes constitutes approx. 3% of the total Zn (Van Wouwe et al., 1990). Further investigation of the role of Zn in apoptosis would be greatly facilitated by a technique, analogous to that for detection of Ca^{2+} by BAPTA analogues and other intracellular fluorophores (Tsien et al., 1982; Tsien, 1989), which has revolutionized our understanding of the role of that cation in cellular events.

Zinquin is the first of a generation of Zn-specific intracellular fluorophores that we have used to detect labile intracellular Zn. It is readily taken up by living cells, is retained for several hours and is essentially non-fluorescent until it complexes Zn. Fluorescence of the Zn–Zinquin complexes in cells can be measured both by spectrofluorimetry and digital image analysis. The latter technique is particularly useful for demonstrating heterogeneity between cells, as was evident in lymphocytes preloaded with Zn. The subcellular localization of the fluorescence can be seen by fluorescence microscopy, although in lymphocytes the fluorescence is weak unless they are preloaded with Zn. In Zn-rich cells such as hepatocytes and pancreatic islet β-cells the fluorescence is very intense without prior loading with Zn (P. D. Zalewski and I. J. Forbes, unpublished work). In all types of cells studied so far, the fluorescence is largely restricted to extranuclear regions. It is not clear whether Zinquin does not localize in the nucleus or whether the labile content of Zn in the nucleus is very low. Because Zinquin is excited by light of relatively low wavelengths (optimal 370 nm), its application to studies involving confocal fluorescence microscopy and fluorescence-activated cell sorting is limited at present by the lack of appropriate lasers on most of these instruments. Because of its relatively low affinity for Zn, Zinquin probably detects only the less tightly bound Zn in cells, including free Zn and Zn loosely-associated with cellular proteins and lipids. The major pool of cellular Zn, which is very tightly bound in active sites of enzymes and in Zn-finger transcription factors, may not be available for interaction with Zinquin. In support of this, our estimations for the amount of labile Zn (14–31 pmol/10^6 cells) detected by Zinquin in the different types of lymphocytes are considerably less than values reported in the literature for total lymphocyte Zn measured by atomic-absorption spectrometry, e.g. 150 pmol/10^6 in peripheral-blood lymphocytes (Cheek et al., 1984). However, we cannot eliminate the possibility that Zinquin may also interact with tightly bound Zn in proteins, provided there is at least one unoccupied ligand-binding site. Thus Zn which plays a catalytic rather than structural role in metalloenzymes contains a potentially free ligand-binding site that is normally only loosely occupied by water (Vallee and Auld, 1990).

Although Zinquin fluorescence in lymphocytes appears weak by microscopy, Zinquin is sufficiently sensitive to signal the relatively small changes of Zn that accompany marked changes in the susceptibility or resistance of these cells to apoptotic DNA fragmentation. The studies here relate to two models of apoptosis: induction of apoptosis in CLL cells by the microtubule poison colchicine (Forbes et al., 1992) and the spontaneous *in vitro* apoptosis of aged thymocytes. Apoptotic death of thymocytes is responsible for the atrophy of the thymus following puberty (Hinsull and Bellamy, 1981). Although in most studies Zn appears to block both DNA fragmentation and cell death, in

some studies only DNA fragmentation was inhibited (see, e.g., Barbieri et al., 1992), indicating that other processes are involved in cell death. Recently, Cohen et al. (1992) showed that Zn blocked apoptosis in dexamethasone-treated thymocytes at an early stage of chromatin condensation, but before DNA fragmentation. A major target for Zn in suppression of apoptosis is probably the Ca^{2+}/Mg^{2+}-dependent endonuclease that mediates internucleosomal DNA degradation (Duke et al., 1983; Giannakis et al., 1991). However, other potential targets include the microtubules, which play an important, although undetermined, role in the regulation of apoptosis (Martin et al., 1991), numerous Zn-finger-containing proteins involved in the apoptotic pathway, including poly(ADP-ribose) polymerase (Rice et al., 1992), protein kinase C (McConkey et al., 1990; Forbes et al., 1992), RP-8 (Owens and Cohen, 1992) and glucocorticoid receptors (Munck and Crabtree, 1981). Zn may also antagonize calcium-dependent processes (Duke et al., 1983), which are essential for induction of apoptosis. In further studies with Zinquin we will correlate levels of labile cellular Zn with changes in activities and subcellular distribution of some of these components.

We are presently using Zinquin to measure the significant fluxes of Zn which are known to occur during early embryonal development (Lutwak-Mann and McIntosh, 1969), in the liver in stress, inflammation, pregnancy and malignancy (Cousins, 1985) and in pancreatic islet β-cells responding to a glucose challenge (Maske, 1957). The implications of these changes in tissue Zn levels for regulation of apoptosis and tissue homoeostasis need now to be determined.

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